NON-HUMAN TRANSGENIC MAMMAL FOR THE CONSTANT REGION OF THE CLASS A HUMAN IMMUNOGLOBULIN HEAVY CHAIN AND APPLICATIONS THEREOF

The present invention relates to non-human mammal transgenic for the constant region of the class A human immunoglobulin heavy chain and to its applications for the production of humanized class IgA antibodies.

The class A immunoglobulins (IgA) comprise two identical heavy chains of isotype α1 (subclass IgA1) or α2 (subclass IgA2) in humans, combined via disulfide bridges with two identical light chains of isotype kappa (κ) or lambda (λ).

The α heavy chain, which is specific to this class of immunoglobulins, exists in membrane form and in secreted form. The secreted form comprises four domains of about 110 amino acids: a variable domain VH and three constant domains CH1, CH2 and CH3, and a hinge (H) region between CH2 and CH3 and a C-terminal octapeptide. The penultimate cysteine of this octapeptide can form a covalent bond with the J chain (or joining piece) which serves to combine two IgA heavy chains so as to form dimeric IgAs. The membrane form additionally comprises a hydrophobic domain allowing anchoring of the protein in the membrane, and an intracytoplasmic domain. The region of the heavy chain corresponding to the CH1, CH2, H and CH3 domains combined either with the C-terminal octapeptide (secreted form) or with the hydrophobic and intracytoplasmic domain (membrane form) is called constant region by contrast to the region corresponding to the variable domain VH which is called variable region.

The κ and λ light chains, which are common to all the classes and subclasses of immunoglobulins, comprise two

domains: a variable domain (VL) and a constant domain (CL). In humans, the expression of the κ and λ chains is equivalent, whereas in mice, the expression of the λ locus is very low such that 95% of the light chains are of the κ type. The region of the light chain corresponding to the CL domain is called constant region by contrast to the region corresponding to the variable domain VL, which is called variable region.

The immunoglobulin genes are organized into loci, one locus for the heavy chains (IgH locus) and one locus for each of the light chains (lambda and kappa loci).

The loci of the light chains each comprise V and J genes encoding the variable domain and C genes encoding the constant domain; during the differentiation of the B lymphocytes, a V gene is rearranged with a J gene and a C gene, and the V region is additionally subjected to somatic mutations which make it possible to produce antibodies with high affinity for the antigen.

The locus of the heavy chains comprises V, D and J genes encoding the variable domain and C (Cμ, Cδ, Cγ, Cε and Cα) genes encoding the constant domains of the isotypes of the different classes of immunoglobulins; each C gene, except Cδ, is preceded by a switch (S) sequence. The Cα (Cα1 and Cα2 in humans) genes contain introns separating the exons encoding the constant domains CH1, CH2 and CH3 and the membrane (mb) exon; the sequence encoding the hinge region is included in the exon cH2. During the differentiation of the B lymphocytes, a V gene is rearranged with a D gene and a J gene, and the V region is also subjected to somatic mutations which make it possible to produce antibodies with high affinity for the antigen. In addition, while the primary response to the antigen mainly consists of IgM, the secondary response is associated with the class switch mechanism during which the switch sequence Sμ, situated upstream of Cμ recombines with another

switch sequence, thus leading to the production of another class of immunoglobulin (IgG, IgE or IgA).

The diversity of the antibodies produced in response to the stimulation by an antigen results from the combination of several mechanisms: the multiplicity of the V genes, the somatic mutation of these V genes, the somatic recombination of the V genes and the somatic recombination of the switch sequences.

The IgAs exist in the body in two different forms: a serum IgA and a secretory IgA (s-IgA).

The serum IgA represents 15 to 20% of the serum immunoglobulins; more than 80% of the human serum IgA is in monomeric form, whereas in most other mammalian species it is essentially in dimeric form.

The secretory IgA constitutes the main immunoglobulin in secretions (ocular, salivary, mammary, tracheo-bronchial and urogenital secretions), where it exists in the form of an IgA dimer combined with another protein, the secretory component, which is probably coiled around the IgA dimer and attached by disulfide bridges to the CH2 domain of each IgA monomer. Unlike the J chain, the secretory piece is not synthesized by the plasmocytes but by the epithelial cells. The dimeric IgA secreted by the subepithelial plasmocytes binds to the poly-Ig receptors present at the basal pole of the epithelial cells. The s-IgA/receptor complex is then endocytosed and transported through the cell while remaining attached to the membrane of the transport vesicles. The latter fuse with the plasma membrane at the luminal surface and release the dimeric IgA combined with the secretory piece which results from the cleavage of the receptor. Thus, the secretory piece facilitates the transport of the IgAs in the secretions and protects them from proteolysis.

Because of their capacity to cross the epithelium of the mucous membranes and to prevent the entry of pathogens such as viruses, bacteria, parasites and toxins, the IgAs play a major role in local immunity: ocular, respiratory, digestive and urogenital immunity. The mode of action of the IgAs encompasses active mechanisms (complement activation, binding to the Fc receptor) and passive mechanisms (blocking of the receptors for pathogens (viruses) and inhibition of the motility of bacteria). A close correlation between a specific IgA response and protection against an infection has been demonstrated, in particular for viruses (rotavirus, influenza virus, poliovirus, cytomegalovirus, respiratory syncytial virus, Epstein-Barr virus). Class IgA protective antibodies directed against numerous human pathogens (HIV, influenza A virus, bacteria, toxins, parasites) have been isolated.

Because of this special property, IgAs have specific applications for the diagnosis and treatment of infectious diseases and cancer. They could be used in passive immunotherapy to neutralize pathogens (serotherapy). They could also be used in active immunotherapy (vaccination) as vector to target tumor antigens or antigens of pathogenic microorganisms in the mucous membranes, so as to induce local immunity specific to these antigens. In addition, they are useful as reliable, safe, stable and well-defined reagent for the diagnosis of diseases such as celiac disease, as a replacement for human IgAs (antitransglutaminase, antiendomysium or antigliadin IgA) obtained from patients, which expose technicians to risks of transmission of human pathogens (virus, prion).

However, the development of these applications is limited because there is no effective method for producing recombinant human or humanized class IgA antibodies.

The expression humanized antibody is understood to mean an antibody derived from a non-human mammal by fusion of the constant domains of the heavy and light chains of a human antibody with the variable domains of the heavy and light chains of an antibody from a non-human mammal.

Indeed, the methods for producing recombinant human or humanized antibodies which are currently available have the following disadvantages:

* the *in vitro* methods are based on the simultaneous expression, from one or more recombinant vectors, of antibody heavy and light chains, of a J chain and optionally of a secretory piece; the heavy and light chains comprise the variable domains of the heavy and light chains (VH and VL) of a human or murine monoclonal antibody of interest, fused respectively with the constant domains CH1, CH2 and CH3 of a heavy chain α, and Cλ or Cκ of a human light chain, or the VH and VL domains are fused with a CH3 domain including the C-terminal octapeptide (International Applications PCT WO 98/30577 and PCT WO 99/54484). For example International Application PCT WO 98/30577 describes the *in vitro* production, with the aid of one or more recombinant baculoviruses, of recombinant human dimeric mini-IgAs (IgA-J) comprising the VH and VL domains of a murine or human monoclonal antibody, each fused with a CH3 domain including the C-terminal octapeptide, combined by means of a J chain; only one recombinant mini-IgA directed against the HIV gp120, obtained from a class IgG1 neutralizing human monoclonal antibody (S1-1 antibody), is described.

These methods, which are specific to IgAs, are limited to murine antibodies and to a few rare human antibodies for which hybridomas have been isolated.

* the *in vivo* methods are based on the production of human monoclonal immunoglobulins from genetically modified mice possessing a transgene consisting of:
- the complete IgH locus and the locus of the kappa light chain, in their germinal configuration, (PCT Application WO 02/059154, Mendez et al., Nature Genetics, 1997, 15, 146-156; Green and Jakobovits, J. Exp. Med., 1998, 188, 483-495 and American Patent Application US No. 08/759,620),
- a mini-IgH locus comprising one or more VH, DH and JH genes, the Cμ gene and a second gene for the constant region, preferably for the Cγ region, and the locus of the kappa light chain (PCT Application WO 02/059154, American Patent US 5,545,807), and
- the complete IgH locus and the locus of the lambda chain in its germinal configuration (American Patent Application US No. 09/734,613). Said mice are optionally genetically disabled for the endogenous kappa locus (κ -/- mice) and optionally possess a mutation which inactivates the endogenous IgH locus (μMT -/- mutation).

These methods do not make it possible to produce large quantities of human class IgA immunoglobulins.

Surprisingly, the inventors have constructed transgenic mouse lines which produce large quantities of humanized class IgA immunoglobulins (in the gram per liter range in mice). The antibodies produced by these animals are predominantly humanized IgAs; they do not contain IgM and only very small quantities of other classes of immunoglobulins (IgG and IgE).

Consequently, the subject of the invention is a non-human transgenic mammal, characterized in that it comprises an IgH locus modified by replacing the switch sequence Sμ with all or part of a transgene consisting of the Cα gene for a human class A immunoglobulin,

including at least the exon encoding the CH3 domain and the membrane exon.

In accordance with the invention, the Cα transgene or the part of this transgene including at least the exon encoding the CH3 domain and the membrane exon, which is inserted in place of the switch sequence Sμ, is therefore located between the intronic activator Eμ, in 5′ and the Cμ gene in 3′ (figure 1).

In this construct, the suppression of the switch sequence Sμ associated with the insertion of the Cα transgene in place of this sequence, abolishes the expression of the endogenous μ gene responsible for the synthesis of heavy IgM chains. In addition, that of the other genes for the immunoglobulin heavy chains is greatly reduced because of the blocking of the class switch toward the immunoglobulin constant genes located downstream of Cμ on the endogenous IgH locus. Thus, the transgenic animals obtained produce large quantities of chimeric IgAs in which the constant domain of the heavy chain is humanized and the variable domains are of murine origin.

The human transgenic α heavy chain benefits from a completely diversified repertoire since it corresponds to the normal repertoire generated by the rearrangements of the VH, D and JH segments of the murine IgH locus. In addition, the transgenic animals are capable of producing antibodies with high affinity as a secondary response to the antigen since their B lymphocytes can recruit the somatic hypermutation phenomenon.

According to an advantageous embodiment of the invention, said non-human transgenic mammal is homozygous for said modified IgH locus.

According to another advantageous embodiment of the invention, said IgH locus is modified by replacing the switch sequence Sμ with the entire Cα gene, including the CH1, CH2, CH3 and mb exons, separated by the corresponding introns.

According to another advantageous embodiment of the invention, said IgH locus is modified by replacing the switch sequence Sμ with the segment of the Cα gene including the exon encoding the CH3 domain and the membrane exon.

According to another advantageous embodiment of the invention, said Cα gene is Cα1.

According to yet another advantageous embodiment of the invention, said non-human transgenic mammal comprises another transgene encoding a human immunoglobulin light chain.

According to an advantageous feature of this embodiment, said light chain is a kappa chain.

Preferably, said transgene is a human kappa gene comprising the intronic activator Eμ upstream and the palindrome *hs3a/hs1,2/hs3b* downstream. These sequences, which are described in Chauveau et al., Gene, 1998, 222, 279-285, make it possible to obtain a high expression of the human kappa chain in B cells and to induce the somatic hypermutation of the human kappa transgene. Preferably, said transgene is under the control of the promoter of the human heavy chain (pVH).

According to another advantageous feature of this embodiment, said non-human transgenic mammal is dizygous for said transgene.

According to an advantageous feature of the preceding embodiments of the invention, said non-human transgenic

mammals comprising another transgene encoding a human kappa light chain possess an endogenous locus of the immunoglobulin kappa light chain inactivated (deleted or mutated) in particular by homologous recombination. Preferably, said non-human transgenic mammals are homozygous for said inactivation; preferably, they are transgenic mice. Among the non-human transgenic mammals in which the endogenous locus of the immunoglobulin kappa light chain has been inactivated by homologous recombination, there may be mentioned in particular the mouse line described in Zou et al., EMBO J., 1993, 12, 811-820.

Such non-human transgenic mammals produce humanized IgAs in which practically all the light chains are of human origin.

According to another advantageous feature of the preceding embodiments of the invention, said non-human mammals transgenic for the α1 heavy chain and optionally for the human kappa light chain possess an endogenous locus of the J chain inactivated (deleted or mutated) in particular by homologous recombination. Preferably, said non-human transgenic mammals are homozygous for said inactivation; preferably, they comprise another transgene encoding a human J chain; more preferably still, they are transgenic mice. Such non-human transgenic mammals are humanized both for the production of IgA and for a protein which combines with the IgAs, the J chain.

The invention encompasses transgenic animals obtained from any mammalian species.

According to another advantageous embodiment of the invention, said non-human transgenic mammal is a transgenic mouse.

The invention encompasses in particular a double-transgenic mouse line, called HAMIGA line for "Humanized Antibodies Made Up Of Monoclonal Immunoglobulin A", comprising:

- an IgH locus modified by replacing the switch sequence Sμ with the Cα1 gene for a human class A immunoglobulin, and
- a complete Vκ gene comprising the rearranged VκI gene with a Jκ5 gene, the Jκ-Cκ intron and the Cκ gene, under the transcriptional control of the promoter of the human heavy chain (pVH), the intronic activator Eμ upstream and the palindrome *hs3a/hs1,2/hs3b* downstream.

The animals of this double-transgenic line produce IgAs that are partially humanized for the heavy chain and completely humanized as regards the light chain.

Indeed, the expression of the transgenic kappa chain in this line is capable of causing allelic exclusion, that is to say of preventing, in most transgenic B cells, the expression of the endogenous genes for murine immunoglobulin light chains.

The repertoire of response to the antigens of this mouse line is normal given that it is mainly the VH domain of the heavy chain which contributes to the formation of the antibody site. Now, the human transgenic α heavy chain benefits from a completely diversified repertoire since it corresponds to the normal repertoire generated by the rearrangements of the VH, D and JH segments of the murine IgH locus, as specified above.

In addition, the mice of this transgenic line are capable of producing antibodies with high affinity as a secondary response to the antigen since their B lymphocytes can recruit the somatic hypermutation

phenomenon both at the level of the gene for the heavy chain and the transgene for the kappa light chain.

The transgenic animals according to the invention are obtained by conventional methods for animal transgenesis, according to the standard protocols as described in *Transgenic Mouse: Methods and Protocols; Methods in Molecular Biology, Clifton, N.J., Volume 209, October 2002, edited by: Marten H. Hofker, Jan Van Deursen, Marten H. Hofker and Jan Van Deursen, published by Holly T. Sklar: Humana Press.*

The sequences of the human and murine genes for immunoglobulins which serve for the construction of the transgenic animals according to the invention are known and accessible in databases. For example, the sequence of CH1, CH2 and CH3 exons and of the membrane exon of the human Cα1 gene correspond to the accession numbers J00220 and M60326, respectively, in the Genbank/EMBL database.

The construction of the Vκ gene is as described in Chauveau et al., Gene, 1998, 222, 279-285; the sequence of the rearranged VκI gene with the Jκ5 gene and the Cκ gene corresponds to the sequence having the accession number X64133 in the EMBL/Genbank database, which encodes a human light chain having the sequence corresponding to the accession number CAA45494 in the EMBL database.

The insertions of gene fragments into the genome of non-human mammals may be carried out in a random manner, preferably they are carried out in a targeted manner, by homologous recombination with an appropriate targeting vector optionally comprising recombination sequences of a site-specific recombinase such as the LoxP sites of the Cre recombinase. The inactivations or deletions of gene fragments in the genome of non-human mammals are carried out by homologous recombination

with an appropriate targeting vector optionally comprising recombination sequences of a site-specific recombinase such as the LoxP sites of the recombinase. The double-transgenic animals are obtained by crossing animals transgenic for the alpha heavy chain with animals transgenic for the light chain, as defined above. The double-transgenic animals are optionally crossed with transgenic animals in which the endogenous locus of the immunoglobulin kappa light chain has been inactivated by homologous recombination and/or with animals in which the endogenous locus of the immunoglobulin J chain has been inactivated and which additionally possess a human J transgene, as defined above.

The subject of the present invention is also a homologous recombination targeting vector, characterized in that it comprises the Cα gene for a human class A immunoglobulin or a segment of this gene including at least the exon encoding the CH3 domain and the membrane exon, flanked by fragments of sequences of the IgH locus from a non-human mammal which are adjacent to the Sμ sequence.

According to an advantageous embodiment of said targeting vector, it comprises a cassette for expressing an appropriate selection marker, adjacent to said Cα gene or to the segment of said gene as defined above.

According to an advantageous feature of this embodiment, said expression cassette is flanked by site-specific recombination sequences. Preferably, said sequences are LoxP sequences of the Cre recombinase. This feature optionally makes it possible to excise said expression cassette.

According to another embodiment of said targeting vector, said fragments of sequences which are adjacent to the Sμ sequence are of murine origin.

According to another embodiment of said targeting vector, the Cα gene or the segment of said gene is flanked in 5′ by a fragment of about 5 kb corresponding to the JH/Eμ region and in 3′ by a fragment of about 5 kb corresponding to the Cμ region, said fragments corresponding respectively to positions 131281 to 136441 and 140101 to 145032 in the sequence of murine chromosome 12 (accession number AC073553 in the EMBL/ Genbank database).

The subject of the present invention is also embryonic cells of a non-human mammal, modified by a targeting vector as defined above.

Said modified embryonic cells (totipotent stem cells) are useful for the production of transgenic mammals as defined above; they are injected into mammalian blastocysts, according to conventional animal transgenesis techniques.

The subject of the present invention is also the use of a non-human transgenic mammal as defined above for the production of humanized class IgA antibodies or fragments of these antibodies.

The subject of the present invention is also a method for preparing humanized class IgA antibodies or fragments of these antibodies, characterized in that it comprises at least the following steps:

- the immunization of a non-human transgenic mammal as defined above with an antigen of interest,
- the production, by any appropriate means, of humanized class IgA antibodies or fragments of these antibodies, from serum, secretions or B lymphocytes

of said non-human transgenic mammal sacrificed beforehand.

The non-human transgenic mammals according to the invention have the advantage of allowing the production of class IgA monoclonal antibodies which are immediately humanized class IgA chimeric antibodies. The method of producing humanized class IgA monoclonal antibodies according to the invention is therefore more simple, more rapid and more economical than the prior art methods since it does not require additional steps of cloning the genes for said antibodies and of fusing the variable domains of said antibodies with the constant domains of human immunoglobulins.

The invention encompasses the production of polyclonal or monoclonal antibodies consisting of monomeric or dimeric IgAs and of s-IgAs, and fragments thereof, in particular the Fab, Fab'2 and Fc fragments.

The humanized class IgA antibodies as defined above and fragments thereof are prepared by conventional techniques known to persons skilled in the art, such as those described in *Antibodies: A Laboratory Manual, E. Howell and D. Lane, Cold Spring Harbor Laboratory, 1988.*

More precisely:

- the polyclonal antibodies are prepared by immunizing a non-human transgenic mammal as defined above with an antigen of interest, optionally coupled to KLH or to albumin and/or combined with an appropriate adjuvant such as Freund's (complete or incomplete) adjuvant or aluminum hydroxide; after obtaining a satisfactory antibody titer, the antibodies are harvested by collecting serum from immunized animals and enriched with IgA by precipitation, according to conventional techniques, and then the specific IgAs are optionally purified by affinity chromatography on

an appropriate column to which the antigen is attached as defined above, so as to obtain a preparation of monospecific IgAs.

- the monoclonal antibodies are produced from hybridomas obtained by the fusion of B lymphocytes from a non-human transgenic mammal as defined above with myelomas, according to the Köhler and Milstein technique (Nature, 1975, 256, 495-497); the hybridomas are cultured *in vitro*, in particular in fermenters or produced *in vivo*, in the form of ascites; alternatively, said monoclonal antibodies are produced by genetic engineering as described in American patent US 4,816,567. For example, non-human transgenic mammals as defined above are immunized strongly and repeatedly with chosen antigens (bacterial, viral or fungal antigens, tumor-specific antigens such as the carcinoembryonic antigen, and the like), according to a standard protocol comprising a first immunization by intraperitoneal injection of the antigen in an equivalent volume of Freund's complete adjuvant and then a second immunization (booster) 15 days later under identical conditions but, this time, with Freund's incomplete adjuvant. The monoclonal antibodies are produced according to a standard protocol comprising sacrificing the animals two weeks after the last booster, removing the spleen, suspending the splenic lymphocytes and fusing these lymphocytes with the SP2/0 cell line (this murine line does not produce any murine antibody, is immortalized, and possesses the entire secretion machinery necessary for the secretion of immunoglobulins).
- the antibody fragments are produced from cloned V_H and V_L regions, from mRNAs for hybridomas and for splenic lymphocytes of an immunized non-human transgenic mammal according to the invention; for example, the Fv and Fab fragments are expressed at the surface of filamentous phages according to the Winter and Milstein technique (Nature, 1991, 349,

293-299); after several selection steps, the antibody fragments specific for the antigen are isolated and expressed in an appropriate expression system, by conventional techniques for cloning and expression of recombinant DNA.

The antibodies or fragments thereof as defined above are purified by conventional techniques known to persons skilled in the art, such as affinity chromatography.

The subject of the present invention is also a humanized class IgA antibody capable of being obtained by the method as defined above, characterized in that it comprises a chimeric heavy chain in which the constant domain(s) are of human origin and a human light chain in which the variable domain is encoded by VκI-Jκ5.

The invention encompasses the humanized class IgA antibodies in which the light chain is encoded by the VκI-Jκ5 gene having the EMBL/Genbank sequence X64133 or a sequence produced by hypermutation of this sequence, in particular after activation of B lymphocytes in the presence of the antigen.

The subject of the present invention is also a fragment of a humanized class IgA antibody capable of being obtained by the method as defined above, characterized in that it comprises a fragment of said heavy and light chains as defined above.

The invention encompasses polyclonal antibodies, monoclonal antibodies and fragments thereof (Fab, Fc, Fab'2).

The humanized antibodies according to the invention and fragments thereof as defined above are well tolerated in humans (minimization of the risk of allergic

reaction by interspecies immunization) and have a prolonged half-life in humans, given that the constant region of the heavy chain and the entire light chain of these antibodies are of human origin.

The subject of the present invention is also a medicament comprising a humanized class IgA antibody or a fragment of this antibody, as defined above; such an antibody or its fragment is used in particular in passive immunotherapy (serotherapy) for the prevention and treatment of an infectious disease or cancer.

The subject of the present invention is also an immunogenic or vaccine composition, characterized in that it comprises at least one humanized class IgA antibody and a fragment of this antibody, as defined above, combined with an antigen, preferably in the form of an antigen-antibody complex comprising a humanized class IgA antibody or a fragment of this antibody directed against said antigen; such a composition makes it possible both to target the antigen to the epithelium of the mucous membranes and to protect it from proteolysis.

The subject of the present invention is also a pharmaceutical composition, characterized in that it comprises at least one humanized class IgA antibody or a fragment of this antibody, as defined above, combined by any appropriate means with an active ingredient; such a composition makes it possible both to target the active ingredient to the epithelium of the mucous membranes and to protect it from proteolysis.

According to an advantageous embodiment of the compositions according to the invention, they additionally contain at least one pharmaceutically acceptable vehicle and optionally carrier substances and/or adjuvants.

The pharmaceutically acceptable vehicles, the carrier substances and the adjuvants are those conventionally used.

The adjuvants are advantageously chosen from the group consisting of oily emulsions, saponin, inorganic substances, bacterial extracts, aluminum hydroxide and squalene.

The carrier substances are advantageously selected from the group consisting of unilamellar liposomes, multilamellar liposomes, miscelles of saponin or solid microspheres of a saccharide or auriferous nature.

The compositions according to the invention are administered by the general route (oral, intramuscular, subcutaneous, intraperitoneal or intravenous) or by the local route (ocular, nasal, vaginal, rectal); the dose and the rate of administration vary according to the species (human or animal) and the disease to be treated.

The subject of the present invention is also a diagnostic reagent comprising a humanized class IgA antibody or a fragment of this antibody, as defined above.

The subject of the present invention is also the use of a humanized class IgA antibody or a fragment of this antibody, as defined above, for the preparation of a medicament intended for the prevention and treatment of infectious diseases and cancer.

The subject of the present invention is also the use of a humanized class IgA antibody or a fragment of this antibody, as defined above, for the preparation of a reagent intended for the diagnosis of infectious diseases and cancer.

In addition to the preceding features, the invention also comprises other features which will emerge from the description which follows, which refers to examples of production and use of non-human transgenic mammals according to the present invention and to the appended drawings in which:

- Figure 1 illustrates the structure of the modified IgH locus obtained by homologous recombination between the murine IgH locus and the targeting vector called p-alpha1KI, comprising a 5.5 kb fragment of the human alpha 1 gene including three exons encoding the constant domains CH1, CH2 and CH3 and the membrane (mb) exon and a neo cassette bordered by LoxP sites (1.6 kb fragment), flanked upstream by a fragment of about 5 kb corresponding to the JH-Eμ region(DQ 52/JH fragment) and downstream by another fragment of about 5 kb corresponding to the Cμ gene (Cμ fragment).
- Figure 2 illustrates the detailed structure of the targeting vector called p-alpha1KI, comprising: a 5.5 kb fragment of the human alpha 1 gene including three exons encoding the constant domains CH1, CH2 and CH3 and the membrane (mb) exon and a neo cassette bordered by LoxP sites (1.6 kb fragment), flanked upstream by a fragment of about 5 kb corresponding to the JH-Eμ region (DQ 52/JH fragment) and downstream by another fragment of about 5 kb corresponding to the Cμ gene (Cμ fragment).
- Figure 3 illustrates the confirmation of the sequence of the targeting vector p-alpha1KI by enzymatic restriction with XhoI. λH3: molecular weight marker. Lanes 3 and 4: clones comprising the neo cassette inserted in the correct orientation; 5 fragments, 2 of which co-migrate (5 kb and 5.3 kb), are detected: 6.4 kb (CH2 + CH3 fragment of α1-neo cassette), 5 kb (Cμ fragment), 5.3 kb (JH fragment + CH1 fragment of α1) and 3.7 kb (plasmid fragment + 5′ DQ52 fragment). Lane 5: clone comprising the neo cassette inserted in the reverse orientation; 4 fragments are detected:

9.5 kb (JH fragment - CH2 + CH3 fragment of α1-neo cassette), 5 kb (Cμ fragment), 3.7 kb (plasmid fragment + 5′ DQ52 fragment) and 2.4 kb (CH1 fragment of α1 + neo cassette).

- Figure 4 illustrates the Southern-blot profile of a recombinant allele, compared with a wild-type allele; the genomic DNA digested with EcoRI is hybridized with a probe located in 5′ of the δ gene.
- Figure 5 illustrates the Southern-blot analysis of the genomic DNA of the ES clones transfected with the targeting vector p-alpha1KI; the genomic DNA digested with EcoRI is hybridized with a probe corresponding to the 5′ region of the δ gene. The arrow indicates a clone which has integrated the human α1 transgene by homologous recombination (7.5 kb fragment corresponding to the recombinant allele and 12 kb fragment corresponding to the wild-type allele).
- Figure 6 illustrates the flow cytometry analysis of the expression of a membrane receptor for the human IgA class at the surface of the peripheral lymphocytes of homozygous animals of the transgenic line alpha1KI. The x-axis represents the labeling with an anti-human α1 antibody labeled with fluorescein and the y-axis represents the labeling with an anti-murine CD19 antibody labeled with phycoerythrin. The dotted rectangle indicates the cells expressing both CD19 (B cells) and a human α1 heavy chain.
- Figure 7 illustrates the flow cytometry analysis of the expression of the human kappa light chain at the surface of the peripheral B lymphocytes of mice of the kappa RNA line, compared with nontransgenic mice (control). The x-axis represents the labeling with the x-axis represents the labeling with an anti-human kappa antibody labeled with fluorescein and the y-axis represents the labeling with an anti-murine kappa antibody labeled with phycoerythrin.
- Figure 8 illustrates the somatic hypermutation of the human kappa transgene in the transgenic mouse line

κ RNA; the distribution of the mutations along the human kappa light chain of 40 clones isolated from B cells activated with PNA was analyzed. The mutations generating an amino acid substitution, the silent mutations and the mutations generating a stop codon are indicated by ■, □, and ▨ respectively. The amino acids corresponding to the sites of hypermutation are indicated by their nature and their position, and by the position of the mutation in the codon (as a Roman numeral, in parentheses).

- Figure 9 illustrates the ELISA analysis of the specific human chimeric IgA1 antibody response in the double-transgenic mice of the HAMIGA line immunized with the ovalbumin antigen. The results are expressed as arbitrary units of anti-ovalbumin IgA.

Example 1: Production and characterization of the transgenic line alpha1KI (alpha1 Knock-In) expressing a chimeric human immunoglobulin alpha 1 heavy chain

The human alpha 1 gene, including the three exons encoding the constant domains CH1, CH2 and CH3 and the membrane (mb) exon, was inserted by homologous recombination, in place of the switch region Sμ of the murine heavy chain (Sμ), so as to block the class switch to the constant genes for immunoglobulins located downstream of Cμ on the endogenous locus (murine IgH locus, figure 1). The targeted region abolishes the expression of the endogenous μ gene responsible for the synthesis of IgM heavy chains, and greatly reduces that of other genes for immunoglobulin heavy chains. Consequently, the transgenic line obtained produces a large quantity of chimeric IgAs in which the humanized constant domain corresponds to the IgA1 isotype.

1) Construction of the homologous recombination targeting vector

The plasmid constructs were produced from the plasmid bluescript SK (pSK) (STRATAGENE) and from the bacterial strain *E.coli* TG1(STRATAGENE), using the conventional protocols for the preparation, cloning and analysis of DNA such as those described in Current Protocols in Molecular Biology (Frederick M. AUSUBEL, 2000, Wiley and Son Inc, Library of Congress, USA).

The homologous recombination vector or targeting vector derived from pSK, called p-alpha1KI (figure 2), comprises: a 5.5 kb fragment of the human alpha 1 gene including three exons encoding the constant domains CH1, CH2 and CH3 and the membrane (mb) exon and a neo cassette (1.6 kb fragment), flanked upstream by a fragment of about 5 kb corresponding to the JH-Eμ region (DQ 52/JH fragment) and downstream by another fragment of about 5 kb corresponding to the Cμ gene (Cμ fragment).

More specifically, the various fragments were inserted into the plasmid bluescript SK, according to the following steps:

In a first step, the Cμ fragment corresponding to positions 140101 to 145032 of murine chromosome 12 (Genbank/EMBL AC073553) was amplified by PCR with the aid of appropriate specific primers and then cloned at the *XhoI* site of pSK to give the plasmid pA.

In a second step, the DQ 52/JH fragment corresponding to positions 131281 to 136441 of murine chromosome 12 (Genbank/EMBL AC073553) was amplified by PCR with the aid of appropriate specific primers and then cloned in 5′ of the Cμ fragment, between the *EcoRV* and *ClaI* sites of the plasmid pA, to give the plasmid pB.

In a third step, the neo cassette described in Pinaud et al., Immunity, 2001, 15, 187-199 was inserted at the

SalI site between DQ52/JH and Cμ, to give the plasmid pC.

The SacI-BamHI fragment of 5.5 kb of a recombinant plasmid comprising the entire human alpha 1 gene, including the exon sequences CH1, CH2 and CH3 (Genbank/EMBL J00220) and the membrane exon (Genbank/EMBL X64133) was ligated at each of its ends with ClaI adaptors.

Finally, in a final step, the 5.5 kb fragment flanked with ClaI adaptors thus obtained was inserted between the JH fragment and the neo cassette at the *ClaI* site of the plasmid pC to give the targeting vector called p-alpha1KI.

The p-alpha1KI sequence was verified by automated sequencing and by restriction analysis with the enzymes *ClaI* and *XhoI* (figure 3).

2) Transfection of ES cells and injection into blastocysts

The clones of ES cells derived from the 129/SJ line were isolated, analyzed and then injected into blastocysts of C57/Black 6 mice using conventional protocols for transgenesis and analysis of genomic DNA, such as those described in Current Protocols in Molecular Biology (Frederick M. AUSUBEL, 2000, Wiley and Son Inc, Library of Congress, USA).

More specifically, ES cells were transfected by electroporation of the p-alpha1KI DNA linearized at the *NotI* site. The clones selected in the presence of geneticin were collected and the genomic DNA digested with *EcoRI* was analyzed by Southern blotting with the aid of a radioactive probe hybridizing outside the site of homologous recombination, in 5′ of the constant Delta (δ) gene and of its *EcoRI* site (figure 4); this

probe, amplified by PCR with the aid of appropriate specific primers, corresponds to positions 140101 to 145032 of the murine chromosome 12 sequence (EMBL/Genbank AC073553).

The presence of a recombinant allele is visualized with a fragment of about 7.5 kb (representing the murine μ fragment and the neo cassette) whereas the wild-type allele corresponds to a fragment of 12 kb (figure 5). Under these conditions, out of 303 clones analyzed, 4 proved positive.

Verification of the karyotype of two of the four recombinant clones showed no chromosomal abnormality (aneuploidy).

These clones were injected into blastocysts of C57/ Black 6 mice using conventional transgenesis protocols such as those described in *Transgenic Mouse: Methods and Protocols,* cited above. Among the mice obtained, those exhibiting the highest degree of chimerism were analyzed by PCR and by ELISA. A mouse line homozygous for the recombinant IgH locus, called hereinafter alpha 1 *knock-in* or *alpha1KI* line, was then obtained by crossing heterozygous animals exhibiting the highest degree of chimerism.

3) Detection of the recombinant IgH locus carrying the human Cα1 gene (alpha 1 *knock-in* or *alpha1KI* allele) and of the wild-type IgH locus (wild-type μ allele) by PCR

The genomic DNA of a tail sample from homozygous animals obtained as specified above was analyzed by PCR with the aid of the following two pairs of primers:

- <u>pair specific for the non-mutated murine IgH locus (wild-type μ allele)</u>:

- ***UpstreamSpe I Smu primer:*** 5' GAG TAC CGT TGT CTG GGT CAC 3'(SEQ ID NO:1)

- ***SacI-3'Imu primer***: 5' GAG CTC TAT GAT TAT TGG TTA AC 3' (SEQ ID NO:2)

The amplification reaction was carried out with a hybridization temperature of 61°C. This PCR amplifies in 30 cycles a fragment of 91 base pairs delimiting the SpeI site specific for the nonmutated murine IgH locus.

- pair specific for the recombinant IgH locus carrying the human Cα1 gene (alpha 1 *knock-in* or *alpha1KI* allele):
- NeoI primer: 5' GCA TGA TCT GGA CGA AGA GCA T 3' (SEQ ID NO:3)
- Neo2 primer: 5' TCC CCT CAG AAG AAC TCG TCA A 3' (SEQ ID NO:4)

The amplification reaction was carried out with a hybridization temperature of 55°C. This PCR amplifies in 30 cycles a fragment of 120 base pairs specific for the recombinant IgH locus carrying the human Cα1 gene (alpha 1 *knock-in* or *alpha1KI* mutation).

A mouse line homozygous for the alpha1KI mutation, called hereinafter alpha 1 *knock-in* or *alpha1KI* line, was established; the animals of this line are systematically and simultaneously negative in PCR with the primers specific for the wild-type μ allele and positive in PCR with the primers specific for the alpha 1 *knock-in* allele.

4) Assay of total serum IgAs by nephelometry and by ELISA

a) nephelometry

The serum IgAs were assayed by nephelometry on an automated mchine BNII™ (BEHRING) using the IgA assay kit (BEHRING), according to the supplier's recommendations.

The assay of the serum IgAs gave results which correlated fully with those of the genotyping carried out by PCR:

- the non-mutant control animals have a zero level of human class IgA immunoglobulins
- the heterozygous animals α1-KI also have an undetectable level of human IgAs and a normal level of murine IgMs
- the homozygous animals α1-KI have a significant level of human IgAs, this level varying between 0.4 and 0.6 g/l in the serum. On the other hand, the murine IgMs are undetectable in the serum of these animals.

b) ELISA

The results obtained by nephelometry were confirmed by ELISA according to the following steps: 96-well plates (Maxisorb™, NUNC) were coated either with non-labeled anti-human IgA antibodies or with non-labeled anti-murine IgM antibodies by incubating overnight at +4°C in the presence of goat Fab'2 anti-human IgAs or anti-murine IgMs (Southern Biotechnologies Associates), diluted 1/500 in 0.1 M carbonate buffer, pH 8.3 (100 microliters/well). After 3 washings with PBS buffer containing 0.1% Tween (PBS-Tween 0.1%), the plates were saturated in the presence of PBS containing 10% fetal calf serum (100 microliters/well). After 3 washings with PBS-Tween 0.1% buffer, the sera to be tested, diluted 1/100 and 1/500 in PBS buffer containing 10% fetal calf serum were added (100 microliters/well) and the plates were incubated for 3 hours at 37°C. After 3 washings with PBS-Tween 0.1% buffer, an anti-human IgA antiserum labeled with alkaline phosphatase or an anti-murine IgM serum labeled with alkaline phosphatase (Biosys) diluted 1/1000 in PBS-Tween 0.1% (100 microliters/well) were added and the plates were incubated for 1 hour at 37°C. After 3 washings with PBS-Tween 0.1% buffer, the bound IgAs and IgMs were visualized by adding alkaline phosphatase substrate (p-

nitrophenyl phosphate, SIGMA) at 1 mg/ml in 0.2M Tris buffer, pH 7.0. The reaction was blocked by adding 0.5N sodium hydroxide (50 microliters/well) and then the absorption was measured at a wavelength of 405 nm.

The quantitative data are obtained by extrapolation with a series for a standard serum (BEHRING) for the assay of the human IgAs, and for a murine monoclonal IgM (SOUTHERN BIOTECHNOLOGIES ASSOCIATES) for the assay of the murine IgMs.

The assay of the serum IgAs by ELISA shows a significant difference between the homozygotes and the heterozygotes; the sera of homozygotes contain 0.4 and 0.6 g/l of IgA1, whereas zero or very low absorbance values are observed for the sera of heterozygotes even at the lowest dilution (1/100). By contrast, when the murine IgMs are assayed by ELISA, a normal murine IgM level is observed (of the order of 1 g/l) in the "non-mutant" control mice and in the animals heterozygous for the α1-KI mutation. On the other hand, the murine IgM level is zero in the animals homozygous for the α1-KI mutation.

5) Investigation of the expression of a membrane receptor for the human IgA class at the surface of the peripheral lymphocytes of mutant animals

The homozygous animals carrying the α1-KI mutation were phenotyped by flow cytometry, by double labeling with the aid of antibodies specific for human IgA1 or murine IgM labeled with fluorescein, and of antibodies specific for B cells (anti-CD19 antibodies) labeled with phycoerythrin. More specifically:

- Preparation of lymphoid cells: two peripheral lymphoid organs: the spleen and the Peyer's patches, were removed separately from homozygous mutant animals α1KI, dilacerated in a versene buffer (Invitrogen), and filtered on sieve (40 microns) in order to obtain a

suspension of individual cells freed of cellular aggregates. The spleen cells were then centrifuged and subjected to an additional step of osmotic shock in order to lyse the red blood cells by resuspending the cellular pellet in 1 ml of distilled water. The cells of the samples were then immediately resuspended in complete medium (RPMI + 10% fetal calf serum), counted and stored on ice.

- Labeling with the aid of fluorescent antibodies: 10^5 cells from each sample were incubated for 30 minutes at 4°C with a 1/100 dilution, either of an anti-mouse IgM antibody labeled with fluorescein isothiocyanate (Southern Biotechnologies), or of an anti-human IgA antibody labeled with fluorescein isothiocyanate, or alternatively the combination of one of the preceding antibodies with an antibody specific for B cells (anti-CD19 antibodies) labeled with phycoerythrin (double labeling). The cells were then washed in 5 ml of PBS and then the supernatant was separated after decantation and the cells were resuspended in 100 microliters of PBS, 0.5% BSA, 0.1 mM EDTA.

- Cytofluorimetric analysis: the labeled cells were analyzed by flow cytometry (COULTER XL™).

The results of the flow cytometry are in agreement with those of the assay of serum immunoglobulins. In the homozygous animals of the alpha1-KI line, no expression of murine IgMs is detected either in the spleen or in the Peyer's patches.

Yet in the absence of expression of IgM, a compartment of CD19+ peripheral B cells is capable of becoming differentiated in these animals and represents 10 to 12% of the spleen lymphocytes or 40 to 60% of the lymphocytes of the Peyer's patches. This compartment expresses membrane IgAs in which the humanized heavy

chain is recognized by an antibody specific for the IgA1s and labeled with fluoroscein (figure 6).

Example 2: Production and characterization of the transgenic line κ RNA expressing a human immunoglobulin kappa light chain

A transgenic animal line expressing in all their B cells a human kappa light chain encoded by the variable region VκI-Jκ5 and the Cκ region (kappa RNA chain, EMBL/Genbank X64133) was obtained by direct transgenesis from the expression vector described in Chauveau et al., Gene, 1998, 222, 279-285.

1) Construction of the transgenesis vector

The transgenesis vector is the plasmid pALIEμ described in Chauveau et al., Gene, 1998, 222, 279-285; it contains both the VH promoter and the Eμ enhancer in 5' of the cassette encoding the kappa RNA chain, and in 3' of this cassette: the three enhancers hs3a, hs12 and hs3b, located in 3'of the IgH locus in 3'. The coding sequence corresponds to the VκI-Jκ5-Cκ chain (Genbank/EMBL X64133). The plasmid pALIEμ was linearized with the restriction enzymes *NotI* and *PvuI* which cut inside the plasmid sequence, *NotI* being located upstream of the promoter which precedes the cloned Vκ segment and *PvuI* being located within the ampicillin resistance gene carried by the plasmid. The fragment including the entire kappa expression cassette flanked by all the promoter and regulatory elements for expression was then randomly inserted into mouse blastocysts using conventional direct transgenesis protocols such as those described in *Transgenic Mouse: Methods and Protocols,* cited above.

2) Identification of the founder animals of the κ RNA line and typing of their progeny

A transgenic mouse line possessing the κ RNA transgene was obtained after injection of the expression vector; the presence of this human transgene was verified on the DNA of the mice by Southern blotting with the aid of a probe specific for the human Cκ region (*EcoRI-EcoRI* fragment of 2.5 kb including the entire human Cκ exon). The animals carrying the insert of the transgene on the two alleles of the site of insertion (homozygous animals) have a double quantity of transgene and can be distinguished by Southern blotting from the animals carrying a single copy of the transgene (heterozygous animals). Alternatively, the presence of the transgene was detected by PCR with the aid of primers which make it possible to specifically amplify the human sequence VκI-Jκ5-Cκ (Genbank/EMBL X64133).

3) Investigation of the expression of the human kappa light chains at the surface of the peripheral lymphocytes of mice of the kappa RNA line

The dizygous animals carrying the kappa RNA transgene were phenotyped by flow cytometry, by double labeling with the aid of an anti-murine κ antibody (labeled with phycoerythrin) in conjunction with an anti-human κ antibody (labeled with fluorescein isothiocyanate), according to the protocol as described in example 1.

These animals show an expression of the human κ transgene on the majority of the B cells (figure 7). Furthermore, the transgene induces a phenomenon of allelic exclusion such that the B cells expressing the human κ transgene do not express an endogenous gene for the mouse light chains. By cytometry, these cells are therefore positive during labeling with the anti-human κ chain antiserum and negative with the anti-murine κ chain antiserum (figure 7).

4) Analysis of the somatic hypermutation of the κ transgene in mice of the kappa RNA line

It has been shown that this human κ light chain is capable of combining with heavy chains and of becoming diversified by virtue of the phenomenon of somatic hypermutation (triggered by a response to the antigen). This transgene which preserves the endogenous architecture of a κ gene with presence of the Jκ-Cκ intron between VκJκ and Cκ, further benefits from a high expression provided by the P_{VH} promoter/Eμ enhancer + regulatory palindrome 3'IgH (hs3a, hs1,2, hs3b) combination. The cumulative action of all these regulatory elements makes it possible to recruit the somatic hypermutation machinery at the level of the transgene. More specifically, the Peyer's patches of transgenic mice are removed by dissection of the intestine. The cellular suspension is prepared by grinding the Peyer's patches through a nylon membrane. The cells are washed three times at +4°C in DMEM containing 10% fetal calf serum. The dead cells were removed after each washing and the cellular suspension was adjusted to 10^6 cells/ml.

The cells were incubated for 30 min at +4°C in the presence of biotinylated anti-B220 antibodies. After two washings with DMEM containing 5% fetal calf serum, the cells were incubated for 30 min at +4°C in the presence of streptavidin coupled to phycoerythrin, and then washed and resuspended in PBS containing 5% fetal calf serum. After adding a lectin specific for the activated B cells (PNA for *peanut agglutinin*) conjugated with FITC, the cellular suspension was incubated for 30 min at +4°C. After two washings with DMEM, the cells were resuspended in DMEM and then they were sorted, by flow cytometry, into two populations: $B220^+PNA^{high}$ (activated B) and $B220^+PNA^{low}$ (resting B).

The genomic DNA was extracted from the two cellular populations sorted with the aid of the kit QIAamp Tissue (QIAGEN). Amplification by polymerase chain reaction (PCR) was carried out on 2 µl of genomic DNA using primers corresponding to the signal region of the human Vκ1 (5'-AAGTCGACATGGACATGAGGGTGCC-3') (SEQ ID NO:5) and at the beginning of the human Jκ5 region (5'-TTCTCGAGACTTAGGTTTAATCTCCAG-3') (SEQ ID NO:6). The amplification program consisted of: an initial step of denaturation at 94°C for 5 min; followed by 35 cycles consisting of a denaturation step at 94°C for 30 s, a hybridization step at 52°C for 30 s and an extension step at 72°C for 30 s; and then a final extension step at 72°C for 7 min.

The amplification product was purified on 1.2% agarose gel, eluted (kitQIAquick Gel Extraction kit, QUIAGEN) and then cloned into the vector pCRII-TOPO (INVITROGEN). The recombinant clones were tested by enzyme restriction and then purified (Flexiprep kit, PHARMACIA) and sequenced by the Sanger method. The sequencing reactions were carried out by PCR with the aid of the primers M13 reverse and M13(-20) and fluorescent dideoxynucleotides and then analyzed by capillary electrophoresis on an automated sequencer (ABI-PRISM 310, PERKIN-ELMER). The sequences obtained from the activated B cells were then aligned with the original sequence of the non-mutated transgene (Genbank/EMBL X64133). The number and the position of the mutations were analyzed (figure 8).

The κ transgene undergoes this somatic hypermutation at a rate which is practically as high (17 mutations per 1000 bases) as the endogenous immunoglobulin genes (which mutate at a rate of 40 mutations per 1000 bases). This single transgene is therefore capable of generating a kappa "repertoire" having some diversity.

Example 3: Production and characterization of the double-transgenic HAMIGA line expressing a chimeric alpha 1 heavy chain and a kappa light chain of human immunoglobulins

The crossing of the κRNA and alpha1-KI lines described in the preceding examples generates double transgenic κRNA/alpha1-KI mice.

To do this, the animals homozygous for the alpha1-KI mutation and homozygous for the κ RNA transgene were crossed with each other. In the first generation (F1) after this crossing, all the animals obtained are heterozygous for the alpha1-KI mutation and heterozygous for the κ RNA transgene. These F1 animals were therefore crossed again: in the next generation (F2) the laws of Mendelian genetics make it possible to obtain 1 animal out of 4 homozygous for the alpha1-KI mutation and one animal out of 4 homozygous for the κ RNA transgene. Among these F2 animals, one animal out of 16 could therefore be selected as carrying both the alpha1-KI mutation in the homozygous state and carrying the κ RNA transgene in the homozygous state. These animals are the founders of the HAMIGA line and they stably transmit to their progeny the genes which simultaneously allow the production of a humanized alpha1 heavy chain in place of the production of murine IgMs and the production and diversification by hypermutation of a human κ chain.

This double transgenic mouse line is called HAMIGA line for *"Humanized Antibodies Made Up Of Monoclonal Immunoglobulin A"*.

1) Production of the double-transgenic HAMIGA line

a) Presence of the κ transgene

The transmission of the κ RNA transgene during crossings of transgenic animals was monitored by

Southern blotting with the aid of a probe specific for the human Cκ region (*EcoRI-EcoRI* fragment of 2.5 kb including the entire human Cκ exon).

The expression of the κ transgene in the mutant animals was detected by ELISA assay of free human kappa chains eliminated in the urine of the animal. More specifically: 96-well plates (Maxisorb®, NUNC) were incubated overnight at +4°C in the presence of a non-labeled anti-human κ antibody (Kallestad) diluted 1/1000 in 0.1M carbonate buffer pH 8.3 (100 microliters/well). After 3 washings with PBS buffer containing 0.1% Tween (PBS-Tween 0.1%), the plates were saturated in the presence of PBS containing 10% fetal calf serum (100 microliters/well). After 3 washings with PBS-Tween 0.1% buffer, the urine samples to be tested, diluted 1/100 and 1/500 in PBS buffer containing 10% fetal calf serum were added (100 microliters/well) and the plates were incubated for 3 hours at 37°C. After 3 washings with PBS-Tween 0.1% buffer, an anti-human κ antiserum labeled with alkaline phosphatase (SIGMA) diluted 1/1000 in PBS-Tween 0.1% (100 microliters/well) was added and the plates were incubated for 1 hour and at 37°C. After 3 washings with PBS-Tween 0.1% buffer, the bound human kappa light chains were visualized by adding alkaline phosphatase substrate (p-nitrophenyl phosphate, SIGMA) at 1 mg/ml in 0.2 M Tris buffer, pH 7.0. The reaction was blocked by adding 0.5N sodium hydroxide (50 microliters/well) and then the absorption was measured at a wavelength of 405 nm.

Alternatively, the expression of the human kappa transgene was analyzed by flow cytometry as described in example 2. The results show that the presence of the κ RNA transgene causes an important phenomenon of allelic exclusion, such that among the peripheral lymphocytes more than 50% express the human light chain

and do not therefore rearrange the genes for the murine light chains in order to express a murine light chain.

b) Homozygosity for the α1-KI mutation

The first single element indicating α1-KI homozygocity is the presence of a high level of human IgA1s in the serum of the animals. In addition, the homozygocity was confirmed by PCR by the positivity of the "α1-KI PCR" combined with the negativity of the "wild-type μ allele PCR". Finally, after sacrificing the animals, flow cytometry analysis made it possible to show on the lymphocytes of the spleen and of the Peyer's patches that the entire B lymphocytes (CD19+) express membrane human IgA1s whereas in parallel no B cell expresses murine IgM.

c) Verification of the simultaneous presence of the alpha1-KI mutation and of the κ RNA transgene in the HAMIGA animals and their progeny

The double-transgenic HAMIGA animals were characterized as those simultaneously corresponding to the two specificities described above: the presence of the κ RNA transgene in the homozygous state and the homozygocity for the alpha1-KI mutation. Furthermore, these animals reproduce while preserving these two specificities and the phenotype of their progeny has the following properties, simultaneously and in a stable manner:

- the production of humanized IgA1 at a sizable level (easily verifiable by ELISA or nephelometry on a simple blood sample taken from live animals at the level of the retro-orbital sinus)
- the production of human κ light chain (easily verifiable by ELISA on a simple urine sample taken from live animals).

2) Immunization of the animals

The animals were immunized once by intraperitoneal injection of 10 micrograms of ovalbumin (SIGMA) diluted in 100 microliters of physiological saline and emulsified with 200 microliters of Freund's complete adjuvant (SIGMA).

After 4 weeks, the animals were subjected to a vaccine booster by intraperitoneal injection of 10 micrograms of ovalbumin (SIGMA) diluted in 100 microliters of physiological saline and emulsified with 200 microliters of Freund's incomplete adjuvant (SIGMA).

3) Assay of the antibodies specific for the vaccine antigen (ovalbumin)

The presence of antibodies specific for the vaccine antigen ovalbumin was analyzed by ELISA 4 weeks, and then 7 weeks after the second injection of the antigen, according to the following technique: 96 well-plates (Maxisorb®, NUNC) were incubated overnight at +4°C in the presence of ovalbumin at the concentration of 10 micrograms/ml in 0.1M carbonate buffer pH 8.3 (100 microliters/well). After 3 washings with PBS buffer containing 0.1% Tween (PBS-Tween 0.1%) the plates were saturated in the presence of PBS containing 10% fetal calf serum (100 microliters/well). After 3 washings with PBS-Tween 0.1% buffer, the serum samples to be tested, diluted 1/20 and 1/100 in PBS buffer containing 10% fetal calf serum were added (100 microliters/well) and the plates were incubated for 3 hours at 37°C. After 3 washings with PBS-Tween 0.1% buffer, an anti-human IgA antiserum labelled with alkaline phosphatase (BIOSYS) diluted 1/1000 in PBS/Tween 0.1% (100 microliters/well) was added and the plates were incubated for 1 hour at 37°C. After 3 washings with PBS-Tween 0.1% buffer, the bound human kappa light chains were visualized by adding alkaline phosphatase substrate (p-nitrophenyl phosphate, SIGMA)

at 1 mg/ml in 0.2M Tris buffer, pH 7.0. The reaction was blocked by adding 0.5N sodium hydroxide (50 microliters/well) and then the absorption was measured at a wavelength of 405 nm. The level of anti-ovalbumin IgA antibodies was expressed as arbitrary units established for sera diluted 1/100 as a function of the Optical density tested serum/Optical density control serum ratio.

The results presented in figure 9 show the presence of antibodies specific for the vaccine antigen ovalbumin 4 weeks (level of human anti-ovalbumin IgA1 antibodies at 388 units), and then 7 weeks after the second injection of the antigen (level of anti-ovalbumin IgA antibodies at 162 units). In parallel, it was also verified that in the absence of immunization of the animals, the level of the anti-ovalbumin IgA antibodies detected remained less than 30 units.

The repertoire of response to the antigens of these mice is expected as subnormal since it is known that it is essentially the VH domain of the heavy chain which contributes to the formation of the antibody site (yet the human transgenic $\alpha 1$ heavy chain benefits from a completely diversified repertoire since it corresponds to the normal repertoire generated by the rearrangements of the VH, D and JH segments of the murine IgH locus). These mice are capable of producing antibodies of high affinity as a secondary response, which results from the fact that their B lymphocytes can recruit the phenomenon of somatic hypermutation both at the level of the heavy chain gene and of the κ RNA light chain transgene.

As is evident from the above, the invention is not at all limited to its embodiments, implementations and applications which have just been described more explicitly; it embraces on the contrary all the variants which may occur to the specialist in this

field, without departing from the framework or the scope of the present invention.